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14. ABSTRACT

We have successfully generated Lentiviruses vector carrying yy1 siRNA (to knock down YY1) or YY1 overexpression cassette. In the cell culture studies, the siRNA YY1 could efficiently knockdown the endogenous YY1 of Pten-/- mouse prostate epithelial cells (MPECs), while the YY1 cDNA could be overexpressed in Pten+/+ MPECs. Before conducting in vivo studies, we wanted to test the effect of YY1 expression to the cell proliferation in vitro using the three dimensional (3-D) collagen culture system, which mimics the in vivo prostate cell growth environment. We observed that the overexpression of YY1 enhanced the cell growth and formation of branching structure of Pten+/+ cells in the 3-D culture system. We used these cells in the renal grafting experiments to study the effect of YY1 expression to the prostate cancer formation in vivo. The renal grafts have been collected and further studies are in the process.

15. SUBJECT TERMS
YY1, Prostate Cancer, Lentivirus, siRNA, knockdown, overexpression

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Table of Contents

Page

A. Introduction:

Prostate cancer (PCa) is a most common cancer in American men. Multiple complex molecular events contribute to the PCa initiation and progression. PCa is characterized by aberrant epigenetics with DNA hypo- and hypermethylation, and altered histone modifications that are known to play key roles in regulating gene expression [1]. The multifunctional protein Yin Yang 1 (YY1) plays an important role in epigenetic regulation of gene expression. YY1 associates with many tumor suppressors, oncogene products and protein modifiers, such as p53, c-myc and Mdm2 [2,3] as well as Ezh2 [4] that mediates histone methylation and has been reported to be involved in PCa progression [5]. Data from our lab and others indicated that YY1 is overexpressed in both human PCa [6] and the PCa of a transgenic mouse model. The goal of this project is to determine the functional role of overexpressed YY1 in PCa. Our overall hypothesis is that overexpressed YY1 is essential to the development and progression of PCa. We will perform *in vivo* studies to test this hypothesis. In addition, we will also investigate how the interplay of YY1-p53-Pten and YY1-Ezh2 contributes to the tumor development of PCa. Our study will improve the understanding of the mechanism that leads to the aberrant epigenetic regulation in PCa. This will provide fundamental support to the development of therapeutic approaches to conquer PCa by reversing its epigenetic abnormality.

B. Body:

Task 1. To generate Lentiviruses expressing YY1 or different siRNAs (**Months 1-8**):

a. Make *yy1* siRNA construct that attenuates overexpressed YY1 of $Pten^{-/-}$ MPECs to a level comparable to that of $Pten^{+/+}$ MPECs (**Month 1-3**).

b. Make YY1 expression constructs that express YY1 in $Pten^{+/+}$ MPECs at a level that is about 5 folds higher than the endogenous YY1 (**Month 1-3**).

c. Transfer the expression cassettes of YY1 and *yy1* siRNA to the Lentivirus vector (LTV-1). Produce the viruses and determine their titers (**Month 4-5**).

d. Examine the YY1 knocking down and YY1 overexpression in the cultured MPECs. Generate stable MPECs expressing *yy1* siRNA, scrambled siRNA, or YY1 (**Month 6-8**).

B.1. Knocking down YY1 expression in $Pten^{-/-}$ mouse epithelial prostate cells (MPECs).

B.1.1. Generation of Lentiviral vectors expressing control and yy1 siRNA.

Since the MPECs always exhibit low efficiency in transfection, we have planned to use Lentiviral vectors to introduce the expression cassettes for yy1 siRNA and control (a scrambled sequence) siRNA.

We modified the previously published Lentivirus vector PLL3.7 [7] by replacing the EGFP cDNA with a red fluorescence protein (RFP) cDNA and creating a new polylinker region. We named the new vector as LTV1-U6-RFP (Figure 1A). The RFP in the new Lentivirus vector will help us to follow up the infected cells in our *in vivo* study, especially in the MPECs that express EGFP. Lentiviral infection is much more efficient than DNA transfection, but it may still generate a mixed cell population if the infection efficiency cannot reach 100%. We previously observed that cells with low YY1 expression exhibited growth disadvantage [2]. To avoid uninfected cell override the YY1 knockdown cells, we also generated a Lentiviral vector that possesses a puromycin (Puro) selection

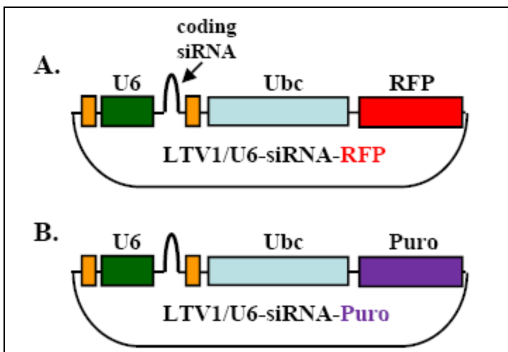

Figure 1. Lentiviral vectors carrying (A) RFP and (B) puromycin resistant gene that are used to introduce siRNA expression cassettes.

mark which allows us to inhibit the growth of uninfected cells. This vector was named as LTV1-U6-Puro (Figure 1B).

The siRNA expression cassettes (driven by U6 promoter) for yy1 and a scrambled sequence constructed by our method [8] were successfully subcloned into these two new Lentiviral vectors to generate LTV1/U6/siRNA-RFP or LTV1/U6/siRNA-Puro constructs (Figure 1). Lentiviruses have been produced by individual co-transfection of these Lentiviral vectors with viral packaging plasmids (VSVG, RSV-REV and PMDL g/p RRE) into 293 cells. Forty-eight hours after transfection, Lentiviral particles were collected from the cultured medium, concentrated by ultracentrifugation (25,000 rpm, 90 min at 4 ºC) and stored in aliquots at -80 ºC. The infection was carried out by incubating Lentivirus with the MPECs at 37 ºC for 6~8 h in the presence of 8 µg/ml of polybrene.

B.1.2. Infecting MPECs with Lentiviruses to knockdown YY1.

We used the produced Lentiviruses to infect the MPECs. We observed that the LTV1/U6-siRNA-RFP based Lentiviruses could infect over 80% of the cells according to the red fluorescent signal visualized in the cells 2-3 days post infection. When cells were collected 3 days post infection and studied by Western blot, YY1 protein could be efficiently knocked down (compare lane 2 with lane 1 in Figure 2). We also observed that Lentivirus carrying a puromycin resistant gene provided better YY1 knocking down effect than that carrying red fluorescent mark (Figure 2, compare lanes 4 with other three lanes).

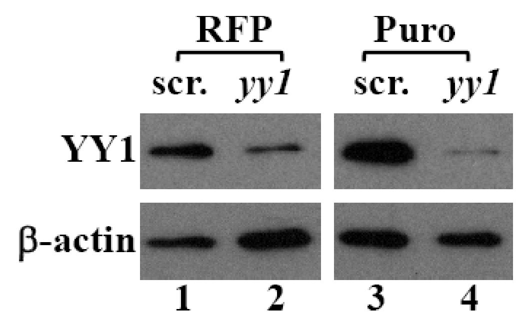

Figure 2. YY1 knocking down in $Pten^{-/-}$ MPECs by Lentiviruses carrying RFP or puromycin resistant gene.
scr.: scrambled sequence.

B.2. Overexpressing YY1 in $Pten^{+/+}$ MPECs

B.2.1. Generating Lentiviral vectors expressing YY1

We have tried to construct an YY1 expressing Lentiviral vector by insert CMV promoter and YY1 cDNA into the LTV-1. However, due to some unknown reasons, the generated vector did not express YY1 efficiently (data not shown). Therefore, we attempted to use another Lentiviral vector to introduce YY1 into MPECs. We tested vectors that are available in Dana-Farber/Harvard Cancer Center DNA Resource Core, Boston. These vectors contain ubiquitin C (Ubc) promoter, PSK promoter and CMV promoter. We modified these vectors in terms of altering the polylinker and fluorescent or antibiotic markers. After subcloning YY1 into these vectors, we found that the Ubc and PSK promoters only gave marginal expression of YY1 in MPECs, but the CMV promoter could present significantly increased YY1 expression. To facilitate our studies, we also generated the CMV-driven YY1 Lentiviral vector with different marker genes, including Zs-Green, RFP and puromycin resistant gene (A, B and C, respectively, in Figure 3). Lentiviruses based on these vectors were produced as described above.

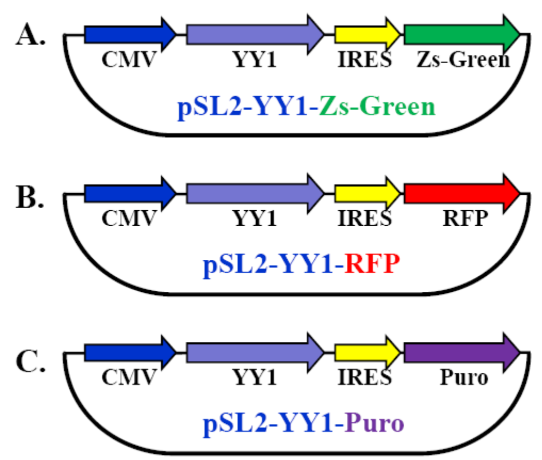

Figure 3. Lentiviral vectors carrying (A) Zs-Green, (B) RFP and (C) Puromycin resistant gene that are used to deliver YY1 cDNA into MPECs.

B.2.2. Infecting MPECs to overexpress YY1

We infected the $Pten^{+/+}$ MPECs with the produced Lentiviruses and observed that over 90% of the cells could be infected based on the green or red fluorescent signal visualized in the cells 2-3 days post infection. The Lentiviral vectors carrying ZsGreen or RFP could lead to a 2-3 fold increase of YY1 (compare lane 2 with lane 1 in Figure 4), while the Lentivirus carrying anti-puromycin gene exhibited much stronger YY1 elevation (compare lane 4 with lane 3 in Figure 4).

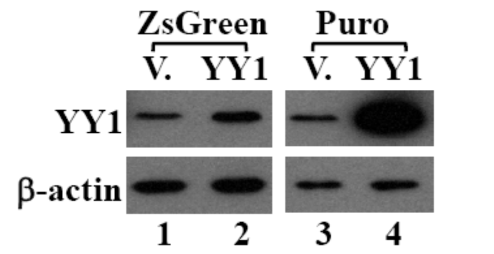

Figure 4. YY1 overexpression in $Pten^{+/+}$ MPECs by Lentiviruses carrying ZsGreen or puromycin resistant gene. **V.**: empty vector control.

B.3. Test whether YY1 knocking down and overexpression will affect the cell growth in vitro

We proposed to carry out in vivo tumor formation studies to test how YY1 expression could affect prostate tumor formation. Before conducting the in vivo studies, we tested the effect of YY1 increase on prostate cell growth using our three-dimensional (3-D) culture system, which can mimic the in vivo growth environment of prostate cells [9]. $Pten^{+/+}$ MPECs were infected by Lentiviruses carrying an empty vector and an YY1 expression cassette, respectively. Two days post infection, aliquots of the transduced cells were collected to confirm YY1 increase by Western blot with β-actin as a control (Figure 5A). The rest of the cells were applied to the 3-D collagen culture system following our published protocol [9]. After 7 days of culture, the wells inoculated by the vector transduced MPECs displayed a limited number of aggregated cell clusters with a few slim branches (Figure 5B, top-left). However, the MPECs transduced by YY1-expressing Lentivirus generated a large number of massively growing and extending ductal structures all over the culture matrix (Figure 5B, top-right). In the immunohistochemical studies, we observed that the dissected branches formed by the YY1-increased MPECs displayed an organized spheroid structure and the cells exhibited strong YY1 staining, but the aggregates grown by the vector-transduced cells lacked this structure (Figure 5C). To quantify the ductal branches and the live cells grown in 3-D culture, we digested the collagen matrix by collagenase and re-

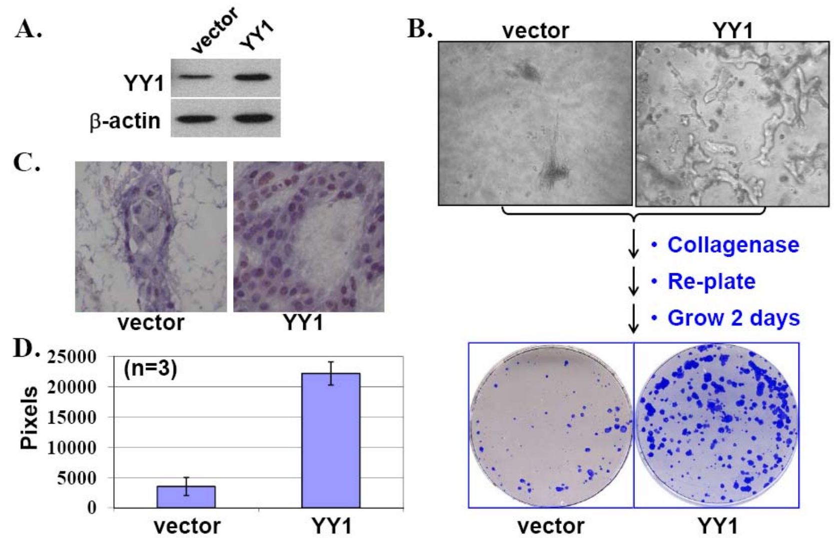

Figure 5. Increased YY1 expression promotes MPEC growth in a 3-D culture system. A. Western blot to detect YY1 and β-actin expression in the transduced MPECs. **B.** Branching structures formed by MPECs (4×10^4 cells/well in a 24-well plate) transduced by Lentivirus carrying empty vector or expressing YY1 (top). The whole collagen culture matrix in each well was the individually digested by collagenase. Pelleted cells were re-plated to 6-cm dishes and stained by crystal violet after 2-day culture (bottom). **C.** YY1 antibody and Hematoxylin co-staining of the dissected ductal branches formed by the MPECs. **D.** Pixels in the dishes of Figure 10B to quantify the branches formed by the MPECs.

cultured the pelleted cells on new dishes for 2 days (Figure 5B). The pixel quantification of the crystal violet stained cells indicated approximately an 8-fold increase of live cells on the dishes originating from the YY1 increased 3-D culture matrix compared to the vector control (Figure 5D). Overall, this study demonstrated that YY1 increase facilitates cell growth in 3-D collagen culture, suggesting that overexpressed YY1 may have a proliferative role in PCa development.

Task 2. To study the effects of YY1 expression to the PCa formation *in vivo* (**Months 9-17**):

a. Start the PCa tumor formation experiments in mice by renal grafting (1) Pten$^{-/-}$ MPECs expressing the scrambled siRNA and *yy1* siRNA; (2) Pten$^{+/+}$ and Pten$^{+/-}$ MPECs containing the YY1 expression cassette and the empty vector (**Months 9-12**).

b. Data analysis of the *in vivo* study, including dissection of the graft, histopathological study, immunohistochemistry and the assessment of the results (Months 13-15).

c. Determine whether YY1 overexpression in PCa is regulated at the transcriptional or posttranslational level (Months 16-17).

B.4. Studying the effect of altered YY1 expression to PCa tumor formation in mice by renal grafting

We have used the Pten$^{-/-}$ MPECs with YY1 knocked down and Pten$^{+/+}$ MPECs overexpressing YY1 to generate renal grafts following a protocol described in Figure 6, which was recently published by our collaborator, Dr. Cramer [10]. We have just collected the renal grafts from the mice and the data analysis of this *in vivo* study, including dissection of the graft, histopathological study, immunohistochemistry and the assessment of the results, is in the process.

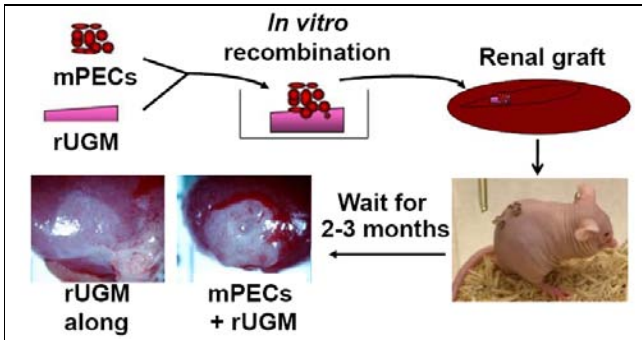

Figure 6. Schematic procedure of renal grafting study.

C. Key Research accomplishment:

1. We have generated different vectors to introduce siRNAs and cDNAs into different cell lines.
2. We observed that YY1 increase could significantly increase the cell growth and branching structure formation in 3-D collagen culture system.

D. Reportable Outcomes:

1. Developed cell lines: Using out lentiviral vectors, we have generated mouse prostate epithelial cell (MPEC) lines: (1) MPEC Pten+/+: overexpress YY1; (2) MPEC Pten-/-: express decreased YY1.

2. Generation of Lentiviral vectors to deliver siRNAs or cDNAs: We have generated different Lentiviral vectors that (1) can be used to deliver siRNAs and cDNAs into different cell lines. These vectors have different marker, including fluorescent proteins (EGFP, Zs-Green and REF/DsRed2) and antibiotic genes (puromycin, neomycin and hygromycin).

3. Funding applied: Based on the in vitro study using the generated MPEC lines mentioned above, we have submitted applications entitled as “The epigenetic role of YY1 in prostate cancer” to NIH/NCI as an R01 application.

E. Conclusion:

We have successfully completed the proposed research in the first 12 months, as described in the **Tasks 1** and **2** of “Statement of Work”.

F. References:

1. Li LC, Carroll PR and Dahiya R. Epigenetic changes in prostate cancer: implication for diagnosis and treatment. **J Natl Cancer Inst**, 2005; 97(2):103-115.
2. Sui G, Affar el B, Shi Y, Brignone C, Wall NR, Yin P, Donohoe M, Luke MP, Calvo D, Grossman SR and Shi Y. Yin Yang 1 is a negative regulator of p53. **Cell**, 2004; 117(7):859-872.
3. Shrivastava A, Saleque S, Kalpana GV, Artandi S, Goff SP and Calame K. Inhibition of transcriptional regulator Yin-Yang-1 by association with c-Myc. **Science**, 1993; 262(5141):1889-1892.
4. Caretti G, Di Padova M, Micales B, Lyons GE and Sartorelli V. The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. **Genes Dev**, 2004; 18(21):2627-2638.
5. Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, Rubin MA and Chinnaiyan AM. The polycomb group protein EZH2 is involved in progression of prostate cancer. **Nature**, 2002; 419(6907):624-629.
6. Seligson D, Horvath S, Huerta-Yepez S, Hanna S, Garban H, Roberts A, Shi T, Liu X, Chia D, Goodglick L and Bonavida B. Expression of transcription factor Yin Yang 1 in prostate cancer. **Int J Oncol**, 2005; 27(1):131-141.
7. Rubinson DA, Dillon CP, Kwiatkowski AV, Sievers C, Yang L, Kopinja J, Rooney DL, Ihrig MM, McManus MT, Gertler FB, Scott ML and Van Parijs L. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. **Nat Genet**, 2003; 33(3):401-406.
8. Sui G and Shi Y. Gene silencing by a DNA vector-based RNAi technology. **Methods in Molecular Biology**, 2005; 309:205-218.
9. Barclay WW and Cramer SD. Culture of mouse prostatic epithelial cells from genetically engineered mice. **Prostate**, 2005; 63(3):291-298.
10. Barclay WW, Axanova LS, Chen W, Romero L, Maund SL, Soker S, Lees CJ and Cramer SD. Characterization of Adult Prostatic Progenitor/Stem Cells Exhibiting Self-Renewal and Multilineage Differentiation. **Stem Cells**, 2008; 26(3):600-610.

G. Appendices:

None.

H. Supporting Data:

All figures and figure legends have been incorporated into the “**B. Body**” of this report.